A Method for Quantitative Determination of Amino Acids

Field of the Invention:

The present invention is directed to methods for the quantitative determination of amino acids.

Summary of the Invention:

The present invention is directed to a novel method for derivatizing amino acids or peptides, which method comprises reacting an amino acid or peptide with a fluorecent benzoxazole derivative, such as 2-chlorobenzoxazole.

The present invention is also directed to a method of detecting, qualitatively and quantitatively, amino acids and peptides, which method comprises reacting an amino acid or peptide with 2-chlorobenzoxazole and detecting the resultant derivative by a suitable method, such as UV or fluorescence methods.

The present invention also relates to the novel benzoxazole- amino acid derivatives, N-(2-benzoxazolyl) amino acids as described herein.

Brief Description of the Drawings:

Figure 1 provides for the results of amino acid separation of N-(2-benzoxazolyl)-amino acids on a Beckman C18 column. The amino acids in the chromatogram were identified by derivatizing and analyzing the commercial amino acids individually.

Detailed Description of the Invention:

Currently, amino acid analysis is routinely performed using HPLC with pre- and post-column derivatization chemistry for enhanced sensitivity. The present invention is directed towards the use of highly fluorescent N-(2-benzoxazole) derivatives, in particular 2-chlorobenzoxazole (CBOX) as a sensitive, fluorescent tag for the quantitative determination of amino acids. CBOX is readily available from commercial sources, such as Aldrich Chemicals.

The present invention is directed towards use of the fluorescent N-(2-benzoxazolyl)-amino acids (BOX-AAs) derivatives in determination of the amino acids by HPLC using pre-column derivatization as described herein. In this procedure, the amino acids are derivatized with the CBOX to yield the highly fluorescent N-(2-benzoxazolyl)-amino acids (BOX-AAs) for detection at very high sensitivity. The derivatives can also be detected using conventional UV detection methods. The BOX-AAs can be separated on a C18 reversed phase columns for quantitative estimation. This method can be used for the preparation of N-(2-benzoxazolyl)-amino acids in large amounts.

Generically the amino acid standards and samples are derivatized with a BOX reagent, preferably CBOX in an alkaline medium, such as sodium carbonate, to yield a stable fluorescent amino acid derivative, which is separated by reversed phase chromatograph (Ultrasphere-ODS, 0.4 x 25 cm, Beckman). All of the BOX-AA are baseline resolved within 35 minutes or so. Peak areas for the BOX-AA are similar except for Pro (45%), Tyr and His (60%) and Lys (200%), presumably due to an additional amine group.

The invention will now be described by reference to the following examples which are merely illustrative and are not to be construed as a limitation of the scope of the present invention. All temperatures are given in degrees centigrade and all solvents are highest available purity.

Experimental

Preparation of the derivatizing solution

An aqueous solution of 0.1-5% sodium acetate tri-hydrate (w/v) was prepared first. Fifty uL of this solution is mixed with 1.0 mL of methanol. 2-Chlorobezoxazole (10-50 uL) was mixed with methanol-sodium acetate solution.

Derivatization of amino acids

Amino acid standards (1.0 nmol each from Pierce) in 50 uL of buffer (for e.g. 0.25 M sodium carbonate) was mixed with 100 uL of the above 2-chlorobezoxazole solution for derivatization. The reaction was allowed to continue typically at 80 oC for 10-60 min. After the reaction, the samples were diluted with sodium acetate solution and an aliquot was injected onto HPLC for analysis.

Proteins were hydrolyzed with 6N hydrochloric acid and the dried hydrolysates were derivatized in a manner similar to amino acid standards.

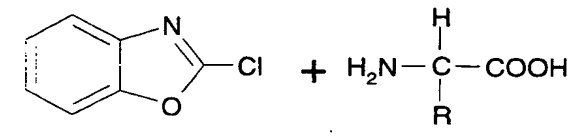
Separation of amino acids

The derivatized amino acids were separated on a C18 reversed phase column. Typical solvent consisted of A: 2% ammonium bicarbonate (0.1-2% w/v) in 20% methanol-water and B: 1-20% methanol in acetonitrile. The amino acids were separated with a gradient generated from these solvents. A typical gradient consisted of 5%B isocratic for 8 min followed by a linear gradient to 45%B over 35 min.

Results

Reaction scheme for amino acid derivatization with 2-chlorobenzoxazole to yield 2-benzoxazole derivatives is shown in Scheme I.

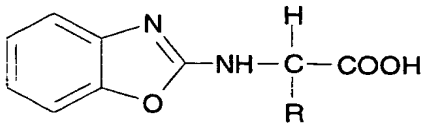
2-Benzoxazole derivatives can be detected using either UV or fluorescence. An excitation and emission maxima of 245 nm and 320 nm respectively were used for fluorescence detection. Separation of BOX-AAs on a Beckman C18 (ODS Ultrasphere, 0.46 x 25 cm) is shown in Figure 1.



2-Chlorobenzoxazole Amino acid

Alkaline Solution

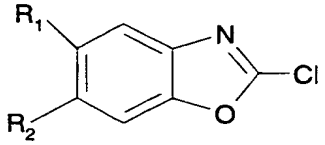
80 °C
10-100 min



N-(2-benzoxazolyl)amino acid
(BOX-AA)

Fluorescence: 245 nm ex. and 320 nm em.

As an alternative to 2-Chlorobenzoxazole, a reagent of formula (I) may also be used:



(I)

wherein R_1 and R_2 are independently selected from the group consisting of hydrogen, halo and C_{1-4}hydroxy.

Scheme I. Reaction scheme for the formation of 2-benzoxazolyl-AAs.

Another aspect of the present invention are the specific derivatized amino acid derivatives with a benzoxazole derivative, i.e., N-(2-benzoxazolyl) amino acids. The commonly used, well known amino acids encompassed by this description include:

Abbreviation Amino acid name

Ala	Alanine
Arg	Arginine
Asn	Asparagine
Asp	Aspartic Acid
Asx	Aspartic Acid or Asparagine
Cys	Cysteine
Glu	Glutamic Acid
Gln	Glutamine
Glx	Glutamine or Glutamic Acid
Gly	Glycine
His	Histidine
Ile	Isoleucine
Leu	Leucine
Lys	Lysine
Met	Methionine
Phe	Phenylalanine
Pro	Proline
Ser	Serine
Thr	Threonine
Trp	Tryptophan
Tyr	Tyrosine
Val	Valine

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

The above description fully discloses the invention including preferred embodiments thereof. Modifications and improvements of the embodiments specifically disclosed herein are within the scope of the following claims. Without further elaboration, it is believed that one skilled in the area can, using the preceding description, utilize the present invention to its fullest extent. Therefore, the Examples herein are to be construed as merely illustrative and not a limitation of the scope of the present invention in any way. The embodiments of the invention in which an exclusive property or privilege is claimed are defined as follows.